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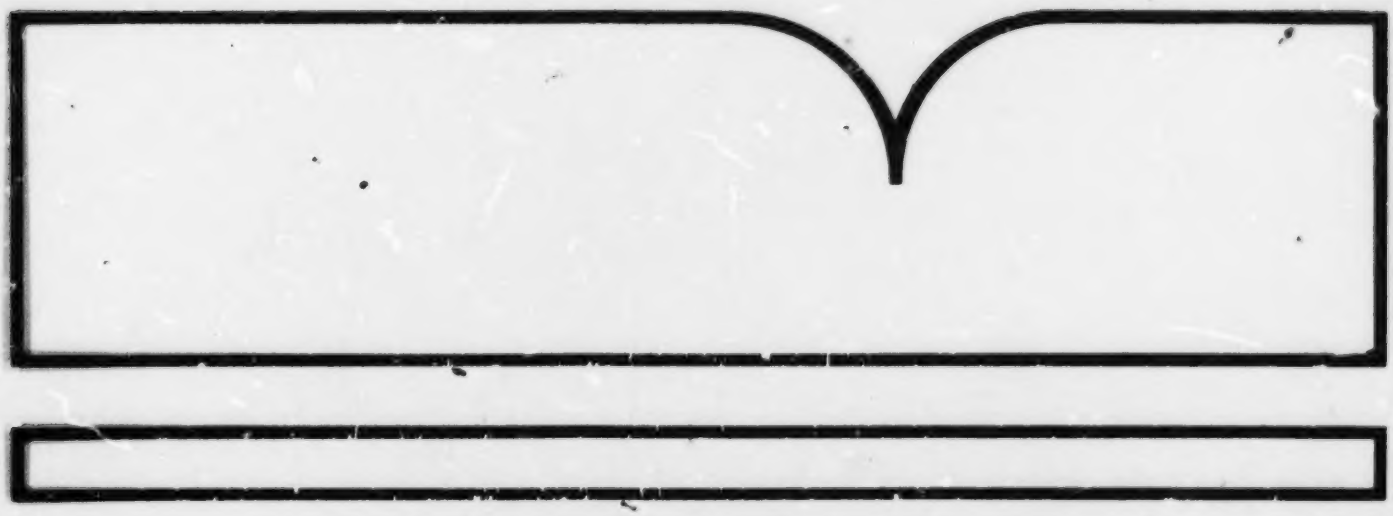

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16. ABSTRACT

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Polymeric Beta-Hydroxyalkanoates from Environmental Samples and *Bacillus megaterium*

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The procaryotic endogenous storage polymer known as poly-beta-hydroxybutyrate is actually a mixed polymer of short-chain beta-hydroxy fatty acids. A method for the quantitative recovery of this mixed polymer, called poly-beta-hydroxyalkanoate (PHA), with analysis by capillary gas-liquid chromatography, showed the presence of at least 11 short-chain beta-hydroxy acids in polymers extracted from marine sediments. Polymers extracted from *Bacillus megaterium* monocultures were also a complex mixture of beta-hydroxy acids with chain lengths between four and eight carbons. Lyophilized sediments were extracted in a modified Soxhlet extractor, and the polymer was purified with ethanol and diethyl ether washes. The purified polymer was treated with ethanol-chloroform-hydrochloric acid (8.5:2.5:1) for 4 h at 100°C, a treatment which resulted in the formation of the ethyl esters of the constituent beta-hydroxy acids. Subsequent assay of the products by gas-liquid chromatography indicated excellent reproducibility and sensitivity (detection limit, 100 fmol). Disturbing sediments mechanically or adding natural chelators increased all major PHA components relative to the bacterial biomass. Gardening of sedimentary microbes by *Clymenella* sp., an annelid worm, induced decreases in PHA, with changes in the relative proportion of component beta-hydroxy acids. The concentration of PHA relative to the bacterial biomass can reflect the recent metabolic status of the microbiota.

The observation of Macrae and Wilkinson (11) that a strain of *Bacillus megaterium* accumulates a polymer called poly-beta-hydroxybutyrate (PHB) under nutrient stress began the investigation into the role of these polymers in the physiology of bacteria. The polymer was thought to be a simple polyester of beta-hydroxybutyrate monomers. The first indication that the polymer was more complex came after the demonstration of two beta-hydroxy fatty acids in polymers isolated from activated sewage sludge (19). Application of quantitative recovery techniques coupled with analysis by capillary gas-liquid chromatography (GLC) in the present study showed that the polymer isolated from marine sediments contained at least 11 constituent beta-hydroxy fatty acids. Polymer extracted from monocultures of *B. megaterium* contained six component beta-hydroxy fatty acid monomers.

A wide variety of procaryotic organisms have been shown to accumulate the polymer. It has been found in numerous heterotrophic and autotrophic aerobic bacteria, photosynthetic anaerobic bacteria (4), gliding bacteria (16), *Actinomycetes* spp. (3, 8), cyanobacteria (2), and, recently, an anaerobic, fatty acid-oxidizing, gram-negative bacterium (12).

In general, monocultures of bacteria and environmental microbial assemblages accumulate the polymer when at least one essential nutrient for growth becomes limiting. For many bacteria, the polymer, once accumulated, serves as the carbon and energy source during starvation. Similarly, it serves as an endogenous source of carbon and energy during encystment and sporulation. For members of the *Azotobacteriaceae*, it appears to function as an electron sink, regulating local oxygen concentrations as well as serving as a carbon reserve (4).

The polymer has been recovered from marine sediments, the epiphytes of sea grasses, and estuarine detritus (6). Electron micrographs have suggested the presence of endogenous storage materials in bacteria recovered from coral reef sediments (13) and groundwater sediments (P. Hirsch and D. L. Balkwill, personal communications). The polymer in the detrital microbiota has been shown to accumulate (biosynthesis with cessation of catabolism) when a nutrient essential for growth is limiting (15). The highest levels of the polymer have been found in microbes from groundwater sediments, suggesting a highly oligotrophic environment (D. C. White et al.; *in* C. H. Ward (ed.), *Proceedings of the First International Conference of Groundwater*

Quality Research, in press).

The analysis of the polymer has classically involved gravimetric determination (10) or the spectrophotometric analysis of *trans*-crotonic acid produced by hydrolysis and dehydration of the polymer (9). These methods are insensitive and subject to error by contamination. Analysis of the constituent beta-hydroxy acids by GLC is an improvement (1). This paper describes a quantitative extraction and purification coupled with analysis by capillary GLC of poly-beta-hydroxyalkanoic acid (PHA) which allowed increased resolution and greatly increased sensitivity. The increased resolution and sensitivity of the GLC analysis showed multiple beta-hydroxy fatty acids from polymers which will be called PHA.

MATERIALS AND METHODS

Materials. Glass-distilled solvents (Burdick and Jackson, Muskegon, Wis.) or freshly redistilled analytical-grade chloroform (Mallinckrodt, St. Louis, Mo.) were used. Derivatizing reagents and authentic standards were purchased from Pierce Chemical Co., Rockford, Ill.; Aldrich Chemical Co., Milwaukee, Wis.; U.S. Industrial Chemicals Co., Tuscola, Ill.; and Sigma Chemical Co., St. Louis, Mo. Fused silica capillary GLC Durabond columns (DB-1) were supplied by J and W Laboratories Inc., Rancho Cordova, Calif.

Polymer standards. *B. megaterium* subsp. *globigii* was grown in nitrogen-free medium (11) modified as described in Herron et al. (6). PHB was isolated from the bacteria by the method of Herron et al. (6).

Sediments. Estuarine sediments were recovered from the Florida State University Marine Laboratory (29°54′ N, 84°37.8′ W). Samples were either frozen in the field with dry ice-acetone or transported in aerated estuarine water to the laboratory for further treatments.

Extraction. Lyophilized sediments were placed in a Soxhlet extractor lined with glass wool and wrapped with a resistance strip heater. Enough chloroform to amply cover the sample was added, and the sample was sonicated for 10 min. The sample was extracted for 2 h in a total of 125 ml of chloroform. The extraction thimble of the Soxhlet extractor was heated so that the chloroform present boiled, maintaining solubility of the polymers. The chloroform was recovered and removed in a rotary evaporator in vacuo.

Purification. The polymer was redissolved in hot chloroform and filtered through a Pasteur pipette plugged with glass wool and heated by a resistance strip heater to 60°C. The chloroform was collected in a screw cap test tube and dried in a stream of nitrogen. The polymer, which was dried to the sides of the test tube, was washed repeatedly with 2 ml of absolute ethanol until the wash was colorless. This process was repeated with diethyl ether. If at any time during the wash procedure polymer was observed lifting off the glass surface, the wash was halted and the sample was redried to the sides of the test tube before the wash was continued.

Derivatization. An internal standard of 0.1 ml of a 0.002 M malic acid solution was added to the purified polymer in the screw cap test tubes. The malic acid was used to monitor the completeness of derivatization and injection volume error. The water was removed in a stream of nitrogen at 40°C. The polymer was dissolved by heating in 0.5 ml of chloroform for 10 min at 100°C. The test tubes were opened while still hot, and 1.7 ml of absolute ethanol and 0.2 ml of concentrated HCl were added. The polymer was cleaved and the ethyl esters of the constituent monomers were formed by heating for 4 h at 100°C. The sample was cooled, and the derivatizing reagents were removed by partitioning against chloroform-water (2:1). Chloroform (2 ml) was added, followed by 4 ml of water. The solution was mixed with a Vortex mixer and briefly centrifuged, and the water was removed. This wash procedure was repeated. The chloroform, which contained the ethyl esters, was transferred to a second screw cap test tube. Two additional chloroform washes were used to ensure quantitative transfer. The collected chloroform washes were dried to 1 drop under a stream of nitrogen at room temperature. Evaporation to dryness led to loss of the volatile ethyl esters. The derivatized hydroxy acids were diluted in a known volume of chloroform for GLC analysis.

Gas chromatographic analysis. The ethyl esters of the constituent monomers and the internal standard were analyzed with a model 3700 gas chromatograph equipped with a CDS 111 controller and a model 8000 autosampler (Varian Associates, Palo Alto, Calif.). Data were processed with a Hewlett-Packard 3502 laboratory data system. A fused silica column (30-m length, 0.25-mm internal diameter) coated with nonpolar silicone SE-30 at a thickness of 0.25 μm was used. The chromatograph was operated in the splitless mode with a 0.5-min venting time. The temperature program was initiated at 45°C for 7 min and then was increased to 200°C at 4°C/min; a 20-min isothermal period followed. The helium carrier gas flow rate was 1.0 ml/min at 1.11 kg/cm^2. The injection port was heated to 220°C and the detector to 250°C. Figure 1 shows a chromatogram of the constituent monomers isolated from an environmental sample.

Mass spectrometry. Capillary gas chromatography-mass spectral (GC-MS) fragmentography was performed with a Hewlett-Packard 5595 A gas spectrometer-mass spectrometer, using the same column and conditions as those used for the GLC analyses. The mass spectrometer was autotuned with decafluorotriphenylphosphine and a 70 × 10^6 eV fragmentation energy. Spectra were recorded at a scan speed of 380 atomic mass units (AMU)/s (4 samples per 0.1 AMU) with a 0.5-s delay between scans of 50 to 500 AMU. The spectrometer was operated in the peakfinder mode at an electron multiplier voltage of 1,800 V. The threshold of detectability was 200 linear counts.

Partitioning of polymers. To attempt to physically separate polymers containing different beta-hydroxy fatty acid monomers, we washed purified polymer from *B. megaterium* and marine sediments five times with 2 ml (each wash) of hot 95% ethanol (19). The ethanol was collected in a screw cap test tube and blown dry under a stream of nitrogen.

Perturbation experiments. To examine the effect of perturbation on the accumulation of PHA in sedimentary microbes, we stirred sediments, stressed them with brown runoff water, and incubated them in the

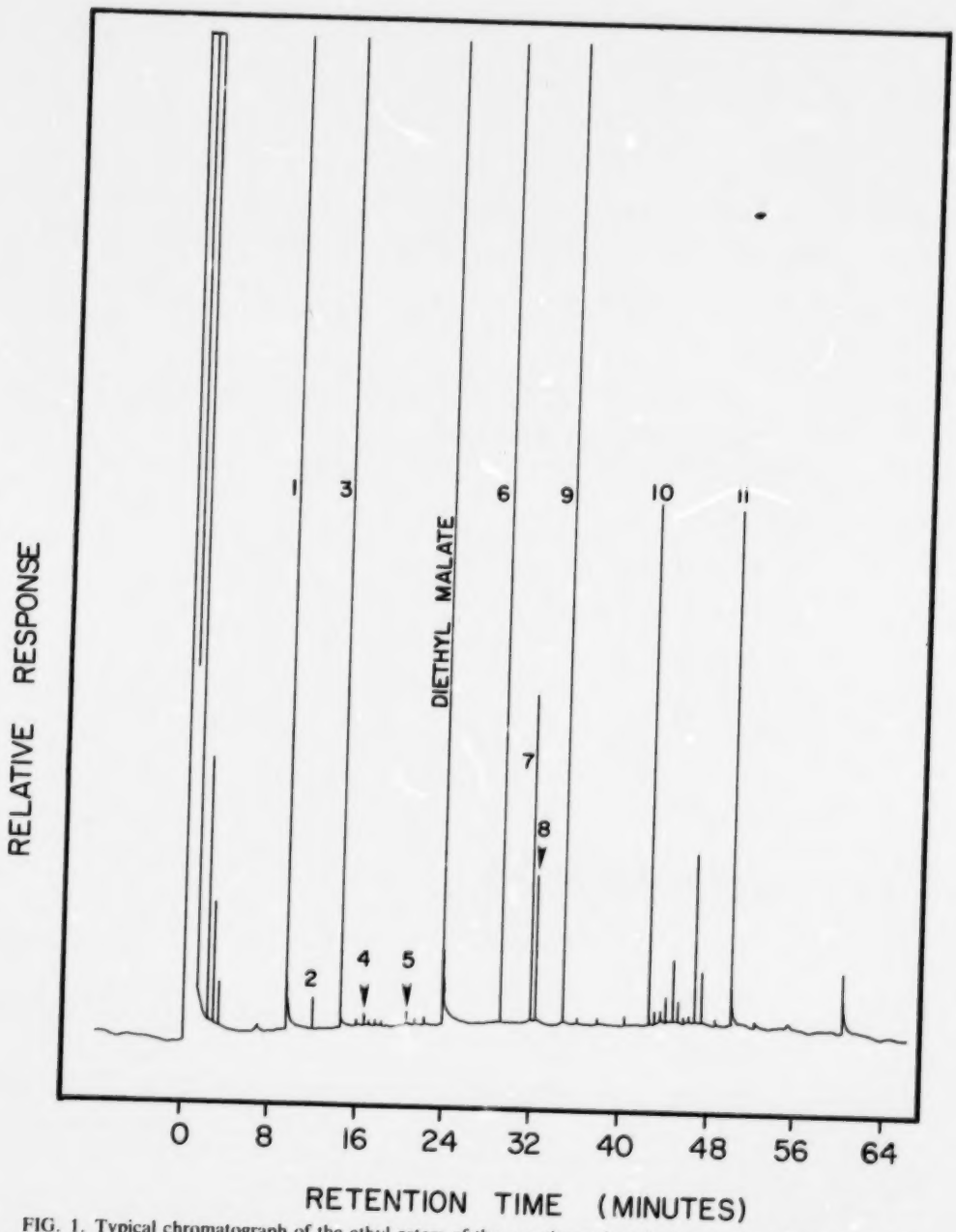
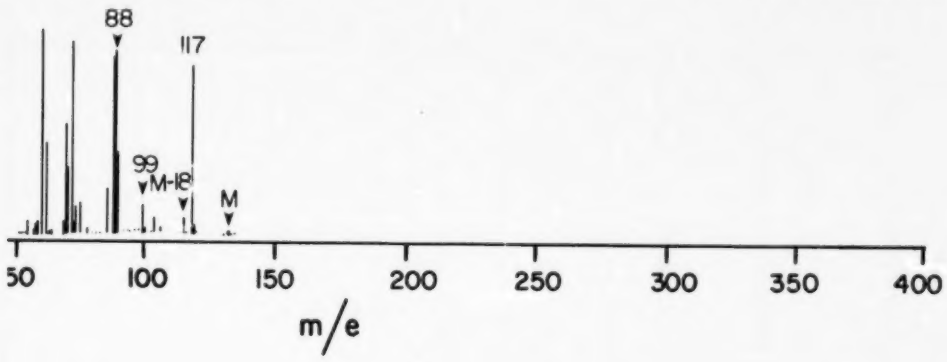
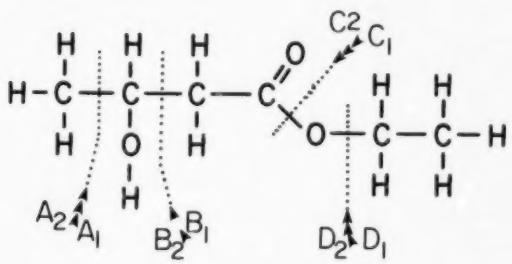


FIG. 1. Typical chromatograph of the ethyl esters of the constituent beta-hydroxy acids of PHA extracted from marine sediments. Known components are as follows: beta-hydroxybutyrate (GC peak no. 1), beta-hydroxyvalerate (GC peak no. 3), and beta-hydroxyheptanoate (GC peak no. 6). Other components with *m/e* values of 117, 99, and 88 in their fragmentation patterns are listed in Table 1 according to the same numbering system.



PROPOSED STRUCTURE



FRAGMENTATIONS

A_2, A_1	15 / 117
B_2, B_1	45/87
C_2, C_1	87/45
D_2, D_1	103/29

FIG. 2. Identification of ethyl-beta-hydroxybutyrate. Upper diagram shows the electron impact mass spectra. Lower diagram shows proposed structure with major fragments (*m/e* values in parentheses) A_1 (117), B_1 (87), C_2 (87), and D_2 (103).

presence of the annelid worm *Clymenella* sp. Sediments used for the mechanical stirring experiment were passed through a 500-μm sieve, and three 500-g samples were placed in 500-ml beakers with 100-ml volumes of estuarine water. One beaker was stirred at 50 rpm, one was stirred at 100 rpm, and one was not stirred. The experiment was destructively sampled after 2 days. Sediments incubated in the presence of brown runoff water were sieved (500 μm), and nine 50-g subsamples were taken. Of these samples, three were frozen, three were incubated with 100-ml volumes of estuarine water, and three were incubated in 75-ml volumes of estuarine water amended with 25-ml volumes of brown runoff water and sufficient sea salts

TABLE 1. Beta-hydroxy fatty acids detected in PHA recovered from an estuarine sediment

GC peak[a]	Retention time (min)	% PHA[b]	Relative abundance of[c]:				Chain length
			117-*m/e* ion	99-*m/e* ion	88-*m/e* ion	M-18 ion	
1	11.2	30.1	82	17	89	9	4
2	13.4	<1	—[d]	—	—	ND[e]	UN[f]
3	16.0	30.5	100	16	31	4	5
4	18.2	<1	—	—	—	ND	5[g]
5	22.6	<1	—	—	—	ND	6
6	30.8	10.0	7	21	3	2	7
7	33.6	2.9	13	44	7	ND	UN
8	34.2	1.4	6	25	10	ND	UN
9	36.4	13.6	17	36	5	ND	7[g]
10	44.4	4.6	2	25	2	ND	8
11	51.6	6.6	5	34	2	2	8[g]

[a] See Fig. 1.
[b] Percentage of PHA was calculated as beta-hydroxybutyrate.
[c] Given as percentage of base peak.
[d] Detected with selective-ion mode; relative abundance unknown.
[e] ND, Not detected.
[f] UN, Chain length unknown.
[g] Preliminary evidence indicates iso-branching.

(Instant Ocean; Aquarium Systems Inc., East Lake, Ohio) to correct the salinity to 22 mg/liter. Samples were incubated with rotation at 100 rpm for 3 days. Sediments used to study the effects of the bamboo worm *Clymenella* sp. were sieved (500 μm), and 10 100-g samples were placed in 10 100-ml beakers. Five beakers were randomly chosen and four bamboo worms were added to each. All beakers were incubated for 3 days in flowing seawater.

Statistics. Significant differences were determined by a two-tailed *t* test. When multiple means were compared, family error rate was controlled by the Newman-Keuls test (14).

RESULTS

Recovery, reproducibility, and sensitivity. Addition of purified polymer to 30 g of lyophilized marine sediments resulted in recovery of 99 ± 3.5% (mean ± standard deviation; $n = 4$) of the added polymer. Multiple analyses of replicate subsamples yielded a reproducibility between 5 and 7%.

Optimum time of extraction was determined to be 2 h for marine sediments. Optimum time of hydrolysis and derivatization was found to be 4 h.

The limit of detectability, measured as a response 2.5 times the background, was found to be 100 fmol of ethyl-beta-hydroxybutyrate. Therefore, samples containing 100 ng of purified polymer could easily be analyzed.

The analysis of a batch of 10 samples required approximately 20 h, 6.5 h of which required the direct attention of the analyst.

Identification of constituent hydroxy acids. GLC-electron impact mass spectrometry of the ethyl ester of beta-hydroxybutyrate showed fragmentation patterns that enabled us to define its structure (Fig. 2). Molecular fragments were detected as follows [data given in the following order—ion, *m/e* (relative abundance)]: M-18, 114 (4); M-44, 88 (89); A_1, 117 (82); A_1-18, 99 (17); B_1, 87 (87); C_2, 87 (87); C_2-18, 69 (54); D_2, 103 (10); D_2-18, 85 (24). These ions accounted for 75% of all major fragments in the mass spectrum. The M-18 and the diagnostic 3,4-cleavage without rearrangement yielding an *m/e* of 117 define the structure as the ethyl ester of a beta-hydroxy acid (18). The mass spectrum of ethyl-beta-hydroxybutyrate derived from the purified polymer was identical to the mass spectrum of the authentic derivative.

Comparison of the spectra of several known beta-hydroxy acids showed that the ions of *m/e* 117, 99, and 88 (a McLafferty rearrangement of a fatty acid ethyl ester) were diagnostic of the ethyl esters of beta-hydroxy acids. Selective ion monitoring of these ions demonstrated the presence of numerous beta-hydroxy acids in the polymers extracted from *B. megaterium* and environmental sediments. Table 1 lists the beta-hydroxy acids present in an environmental sample and retention time, chain length (when known), and relative abundances of M-18 and the ions of *m/e* 117, 99, 88. Plots of log retention time versus carbon number revealed at least two homologous series of beta-hydroxy acids: straight-chain hydroxy acids and what were most likely branched-chain beta-hydroxy acids (Fig. 3). Since the branched-chain homologs fall on a line that includes a six-carbon ester which shows a prominent fragmentation at *m/e* 117, they are likely to be iso-branched. Prolongation

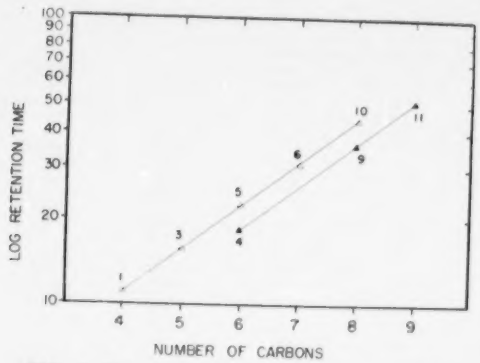


FIG. 3. Plot of retention time (minutes) versus carbon number. Symbols: △, straight-chain beta-hydroxy acids; ▲, what are believed to be iso-branched beta-hydroxy acids. Numbers indicate beta-hydroxy acids shown in Fig. 1 and Table 1.

of the GLC analysis for an additional 100 min at 200°C showed no beta-hydroxy acid components other than those listed in Table 1.

Composition of polymers. Purified polymers from *B. megaterium* contained approximately 95% beta-hydroxybutyrate, 3% beta-hydroxyheptanoate, 2% beta-hydroxy acid no. 10, and trace amounts of beta-hydroxyvalerate, beta-hydroxy acid no. 2, and beta-hydroxy acid no. 4 (numbers indicate peaks as given in Fig. 1 and Table 1). Compositions of polymers extracted from environmental samples were variable, with beta-hydroxybutyrate and beta-hydroxyvalerate being the primary constituents. Table 1 lists the composition of a typical environmental polymer. The compositions were calculated with the assumption that the responses from the flame ionization detector to all constituent fatty acids were equivalent to the beta-hydroxybutyrate standard.

Separation of polymers. Purified polymers were washed with hot 95% ethanol in an attempt to segregate the polymers containing different beta-hydroxy fatty acids, as described previously (19). The combined ethanol washes of the polymer from *B. megaterium* contained less than 1% of the beta-hydroxybutyrate, beta-hydroxyheptanoate, and beta-hydroxy acid no. 10 and 25% of the beta-hydroxyvalerate present in the original polymer. The combined ethanol washes of the environmental polymer contained less than 2.5% of all constituent hydroxy acids recovered from the hot chloroform-soluble fraction.

Effects of perturbations. Sediments mechanically stirred showed lower extractable lipid phosphate (31 μmol/g [dry weight]) than did unstirred controls (50 μmol/g [dry weight]). Mechanically stirred sediments showed increased PHA (425.3 μmol/g [dry weight]) compared with unstirred sediments (398.1 μmol/g [dry weight]). Field samples taken in January 1982 contained no polymer (Table 2). The disturbance of sieving the sediments and shaking them at 100 rpm for 3 days resulted in the production of 1.6 μmol of polymer-bound beta-hydroxy acids per g (dry weight). The addition of brown runoff water, a stress known to induce polymer formation (15), tripled polymer production.

The polymer extracted from sediments that contained *Clymenella* sp. showed total PHA in decreased yield and with different proportions of constituent beta-hydroxy acids, compared with polymers extracted from sediment that did not contain *Clymenella* sp. (Table 3).

DISCUSSION

Improvements in sensitivity and resolution described above led to the detection of 11 constituent beta-hydroxy fatty acids in PHA recovered from marine sediments. Six of these beta-hydroxy acids were found to be components of PHA extracted from *B. megaterium*. Each of these components must have been covalently linked to the hot chloroform-soluble polymer to have been recovered in this analysis. All diethyl ether-soluble lipids were removed from the polymer before ethanolysis. The polymer that has in the past been called PHB should be called PHA.

Not only have new component monomers been detected by these methods, but the use of

TABLE 2. Effect of perturbations on polymer production in marine sediments

Perturbation	Polymer production (μmol/g [dry wt])[a]			
	β-Hydroxybutyrate	β-Hydroxyvalerate	PHA[b]	Lipid phosphate
Sieved sediment control	<0.001	<0.0001	<0.001	10.1 (1.1)
Agitated suspension	0.42 (0.21)	0.96 (0.23)	1.61 (0.32)	14.3 (2.5)
Agitated suspension with humic acids	1.55 (0.04)	1.33 (0.42)	4.30 (0.38)	16.0 (1.2)
Significance	$P = 0.01$[c]	$P = 0.05$[c]	$P = 0.01$[c]	

[a] Mean (± standard deviation); $n = 3$.
[b] Sum of all polymer-bound beta-hydroxy acids.
[c] Significance when agitated suspension was compared with agitated suspension with humic acids.

TABLE 3. Effect of a bamboo worm (*Clymenella* sp.) on endogenous storage polymers

Type of sediment	Polymer production (μmol/g [dry wt] of sediment)[a]			
	β-Hydroxybutyrate	β-Hydroxyvalerate	PHA[b]	Lipid phosphate
Without *Clymenella* sp.	8.6 (2.0)	11.0 (1.5)	31.1 (3.7)	13.4 (0.4)
With *Clymenella* sp.	8.2 (1.8)	6.5 (2.2)[c]	24.9 (2.1)[c]	13.0 (0.6)

[a] Mean (± standard deviation); $n = 5$.
[b] Sum of all polymer-bound beta-hydroxy acids.
[c] $P = 0.05$.

hot concentrated sulfuric acid is avoided, and samples containing chromophores that have absorbance spectra at 235 nm, such as humic acids and tannins, can be successfully analyzed. If mass spectrometry is coupled to it, GLC analysis of component beta-hydroxy acids can be used to estimate incorporation of ^{13}C from ^{13}C-labeled precursors. The high specific activities of the ^{13}C-labeled precursors and the sensitivity of their detection in the component beta-hydroxy fatty acids make possible exposures to labeled-precursor concentrations just above the ambient levels.

The compositions of environmental polymers were observed to be variable and to shift with incubation conditions. All attempts to determine if only one type or a series of endogenous storage polymers with different structures exist have been inconclusive. Attempted differential solubilization (19) yielded a subfraction of extracted polymer that did not differ significantly in component beta-hydroxy acids from the original polymer. It is possible that the ethanol soluble subfraction is comprised of polymers containing fewer monomers than the ethanol-insoluble polymers. Preliminary results indicate that all endogenous storage polymers based on beta-hydroxy acids are heteropolymers. A survey of polymers extracted from bacterial monocultures and environmental sources is under way to determine if a homopolymer of beta-hydroxybutyrate exists.

Synthesis of endogenous storage polymers from acetate, butyrate, and hydroxybutyrate is well documented (4), but the detection of odd-chain-length beta-hydroxy acids in endogenous storage polymers suggests that there are at least two sources of the monomers. The beta-oxidation of odd-chain-length fatty acids has been suggested as a source of the odd-chain-length beta-hydroxy acids incorporated into PHA (12, 19).

In agreement with previous studies, mechanical disturbance of the sediments and treatment with brown runoff water resulted in increased production of PHA (Table 2). Mechanical disturbance disrupts the redox potential of the sediments (6), and brown runoff water behaves much like the chelator EDTA (15). Such perturbations could increase the concentration of reduced carbon relative to concentrations of essential nutrients, thus inducing unbalanced growth. A particularly important environment where unbalanced growth conditions occur is groundwater sediment (White et al., *in* C. H. Ward (ed.), in press).

In contrast to the other perturbations, the presence of *Clymenella* sp. in sediments produced a decrease in PHA. The activities of this bamboo worm disrupted the sediment, increasing the availability of reduced carbon in the aerobic portion of the sediments. This induced a PHA-to-bacterial biomass ratio that differed from that induced by mechanical disturbance alone. These worms may increase local concentrations of all essential nutrients (7, 17). Relative PHA levels have been shown to decrease in detritial microbes under balanced growth (14). The actions of *Clymenella* sp. appear to maintain balanced growth in the sediments that it gardens.

The formation and breakdown of PHA can be important in defining the recent nutritional history of the microbial community (15). The two parameters that define the role of microbes in nature are biomass and metabolic activity (D. C. White, Symp. Soc. Gen. Microbiol., in press). Multiple measures of biomass are available (20; R. H. Findlay, D. W. Moriarty, and D. C. White, Geomicrobiol. J., in press). However, measurement of metabolic activity often involves a disturbance during the exposure of the substrate whose metabolism is to be studied. This disturbance may very likely be responsible for most of the activity measured and may yield artifactual results. Measurements of recent nutritional status, such as triglyceride levels in eucaryotes (5) or PHA accumulation as seen in the present study, may offer a method of defining possible effects of disturbance during measurements of metabolic activity.

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LITERATURE CITED

1. **Braunegg, G., B. Sonnleituer, and R. M. Lafferty.** 1978. A rapid gas chromatographic method for the determination of poly-beta-hydroxybutyric acid in microbial biomass. Eur. J. Appl. Microbiol. Biotechnol. **6**:20–37.
2. **Carr, N. G.** 1966. The occurrence of poly-beta-hydroxybutyrate in the blue-green alga *Chlorogloea fritschii*. Biochim. Biophys. Acta **120**:308–310.
3. **Davis, J. B.** 1964. Cellular lipids of a *Nocardia* grown on propane and *n*-butane. Appl. Microbiol. **12**:301–304.
4. **Dawes, J. B., and P. J. Senior.** 1973. The role and regulation of energy reserve polymers in microorganisms. Adv. Microb. Physiol. **19**:135–278.
5. **Gehron, M. J., and D. C. White.** 1982. Quantitative determination of the nutritional status of detrital microbiota and the grazing fauna by triglyceride glycerol analysis. J. Exp. Mar. Bio. Ecol. **64**:145–158
6. **Herron, J. S., J. D. King, and D. C. White.** 1978. Recovery of poly-β-hydroxybutyrate from estuarine microflora. Appl. Environ. Microbiol. **35**:251–257.
7. **Hylleberg, J.** 1975. Selective feeding by *Abarenicola pacifica* with notes on *Abarenicola vagabunda* as a concept of gardening in lugworms. Ophelia **14**:113–137.
8. **Kannan, L. V., and Z. Rehacek.** 1970. Formation of poly-beta-hydroxybutyrate by Actinomycetes. Indian J. Biochem. **7**:126–129.
9. **Law, J. H., and R. A. Slepecky.** 1961. Assay of poly-β-hydroxybutyric acid. J. Bacteriol. **82**:33–36.
10. **Lemoigne, M.** 1926. Produits de déshydration et de polymérisation de l'acid beta-oxybutyrique. Bu!l. Soc. Chim. Biol. **8**:770–782.
11. **Macrae, R. M., and J. F. Wilkinson.** 1958. Poly-beta-hydroxybutyrate metabolism in washed suspensions of *Bacillus cereus* and *Bacillus megaterium*. J. Gen. Microbiol. **19**:210–222.
12. **McInerney, M. J., M. P. Bryant, R. B. Hespell, and J. W. Costerton.** 1981. *Syntrophomonas wolfei* gen. nov. sp. nov., an anaerobic, syntrophic, fatty acid-oxidizing bacterium. Appl. Environ. Microbiol. **41**:1029–1039.
13. **Moriarty, D. J. W., and A. C. Hayward.** 1982. Ultrastructure of bacteria and the proportion of gram negative bacteria in marine sediments. Microb. Ecol. **8**:1–12.
14. **Myers, J. L.** 1979. Fundamentals of experimental design. Allyn and Bacon, Inc., Boston.
15. **Nickels, J. S., J. D. King, and D. C. White.** 1979. Poly-β-hydroxybutyrate accumulation as a measure of unbalanced growth of the estuarine detrital microbiota. Appl. Environ. Microbiol. **37**:459–465.
16. **Pringsheim, E. G., and W. Wiessner.** 1963. Minimum requirements for heterotrophic growth and reserve substance in Beggiatoa. Nature (London) **197**:102.
17. **Rhoads, D. C., R. C. Aller, and M. B. Goldhaber.** 1977. The influence of colonizing bethos on physical properties and chemical diagenesis of the estuarine seafloor, p. 113–138. *In* B. C. Coull (ed.), Ecology of marine benthos. University of South Carolina Press, Columbia.
18. **Ryhage, R., and E. Stenhagen.** 1960. Mass spectrometric studies. VI. Methyl esters of normal chain oxo, hydroxy, methoxy, epoxy-acid. Ark. Kemi **37**:545–574.
19. **Wallen, L. L., and W. K. Rohwedder.** 1974. Poly-beta-hydroxyalkanoate from activated sludge. Environ. Sci. Technol. **8**:576–579.
20. **White, D. C., W. M. Davis, J. S. Nickels, J. D. King, and R. J. Bobbie.** 1979. Determination of the sedimentary microbial biomass by extractible lipid phosphate. Oecologia **40**:51–62.